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Roles of phospholipase C β2 in chemoattractant-elicited responses

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ABSTRACT **The physiological roles of phospholipase C (PLC) β2 in hematopoiesis, leukocyte function, and host defense against infection were investigated using a mouse line that lacks PLC β2. PLC β2 deficiency did not affect hematopoiesis, but it blocked chemoattractant-induced Ca^{2+} release, superoxide production, and MAC-1 up-regulation in neutrophils. In view of these effects, it was surprising that the absence of PLC β2 enhanced chemotaxis of different leukocyte populations and sensitized the *in vivo* response of the PLC β2-deficient mice to bacteria, viruses, and immune complexes. These data raise questions about the roles that PLC β2 may play in signal transduction induced by chemoattractants in leukocytes.**

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce two important second messengers, inositol trisphosphates and diacylglycerol (1). There are four different PLC β isoforms that have been cloned. They are all regulated by heterotrimeric G proteins, and there is evidence suggesting that different isoforms may be involved in a variety of signaling circuits. The β2 isoform is found primarily in hematopoietic cells (2, 3), and it can be activated by both the Gα subunits of the Gq class and by the βγ subunits generated by a number of different heterotrimeric G proteins (3–9). Cotransfection experiments in COS-7 and HEK cells suggest that PLC β2 may function downstream of chemoattractant receptors. Transfection of receptors for complement component C5a and fMet-Leu-Phe (fMLP) (10), interleukin (IL)-8 receptors a and b (11), and CKR-1 and -2 (12) demonstrated that each of the receptors activates PLC β2 through the pertussis toxin (PTx)-sensitive release of βγ from the G_i class of heterotrimeric G proteins. In addition, this may be a primary signaling pathway in neutrophils, because much of the PLC activity elicited through chemoattractant receptors also appears to function through the G_i-mediated release of βγ (13–17).

To confirm the existence of the Gβγ–PLC β2 pathway *in vivo* and to investigate the function of the pathway in hematopoiesis and leukocyte function, we generated a mouse line that lacks PLC β2. We found that PLC β2 is the major isoform that mediates PTx-sensitive PLC activation induced by chemoattractants and that PLC β2 is critical to many chemoattractant-elicited responses, including Ca^{2+} efflux, superoxide production, and up-regulation of MAC-1. However, PLC β2 deficiency does not attenuate chemoattractant-induced chemotaxis; surprisingly, it was found to enhance the process.

MATERIALS AND METHODS

Generation of PLC β2-Null Mice. An 8-kb genomic DNA from a 129SV agouti mouse strain library contains two exons of the PLC β2 gene, and it was used to make the gene-targeting construct. The exons encoded residues 378–464, which are located in the C terminus of the X box. Parts of the exons were replaced with a neomycin-resistance gene. The gene-targeting construct was transfected into embryonic stem (ES) cells (CJ7 clone) by electroporation. After selection with Geneticin, eight ES clones were obtained in which one of the PLC β2 genes was disrupted. Two of the ES cell clones were microinjected into blastocysts, and eight chimeras were generated with chimerism ranging from 40% to 95%. These chimeras were then backcrossed with 129SV to generate inbred heterozygotes. Finally, interbreeding of heterozygous siblings yielded animals homozygous for the desired mutation—i.e., mice lacking PLC β2. The animals are maintained under specific pathogen-free conditions.

PLC Assays. Neutrophils used for PLC assays were isolated from mouse bone marrow by using a neutrophil isolation kit from Cardinal Associates (Santa Fe, NM). Cells were labeled overnight with [^{3}H]inositol at 5 μCi/ml (1 μCi = 37 kBq), and the levels of inositol phosphates were determined as previously described (18).

Ca^{2+} Efflux, Superoxide Production, and Surface MAC-1 Expression Assays. Mice (8–10 weeks old) were injected intraperitoneally with 2 ml of 2% casein (19). After 3 hr the peritoneal cavities were lavaged with Hanks' balanced salt solution (HBSS), and the peritoneal exudate cells (PEC) were collected by low-speed centrifugation. The percentage of neutrophils was determined by cytospin differential count; typically the recovered cells were >90% neutrophils. The cells were spun down and resuspended in HBSS and were ready for the assays. In the Ca^{2+} assays, PEC were loaded with Fura-2/AM (1 μM) in HBSS at 37°C for 30 min and washed twice with HBSS. The cells were resuspended in HBSS, and the 340/380-nm excitation ratio (emission = 510 nm) of the cells in the absence and presence of ligand was determined with a fluorometer as directed by the user manual (Quantamaster, Photon Technology International, Princeton, NJ). The intracellular [Ca^{2+}] was calculated on the basis of a calibration scale generated by using Fura-2 free acid (20).

The levels of superoxide anion in neutrophils were determined as previously described (21). Briefly, PEC were primed with or without 0.2 μg/ml lipopolysaccharide (LPS) at 37°C for 15 min. A cocktail of *p*-hydroxyphenylacetate (10 mg/ml), superoxide dismutase (1 mg/ml), and horseradish peroxidase (8 mg/ml) was made up in a volume ratio of 25:40:10, and 20 μl was used per 100 μl of cell suspension. Cells were incubated with the cocktail in either the absence or the presence of chemoattractant fMLP (1 μM) at 37°C for 60 min. The cells were excited at 323 nm, and the emission at 400 nm was

Abbreviations: PLC, phospholipase C; fMLP, fMet-Leu-Phe; IL, interleukin; PTx, pertussis toxin; MBSA, methylated bovine serum albumin.

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measured. The superoxide anion concentrations were calculated on the basis of a standard curve generated with known concentrations of hydrogen peroxide.

Surface expression of MAC-1 on neutrophils was assessed by incubating cells with 1 μM fMLP for 5 min at 37°C and then incubating with fluorescein isothiocyanate-conjugated rat anti-MAC-1 antibody or rat isotype-matched control antibody on ice for 30 min. MAC-1-positive cells were identified in a gated population based on forward and side scatter profiles. Flow cytometry was performed using an Elite Flowcytometer (Coulter). Data were acquired with 10,000 cells per sample and expressed as the percentage of maximal mean channel fluorescence minus that obtained with the control antibody.

Chemotaxis Assay. Bone marrow cells were freed of red blood cells by hypotonic lysis, and the $CD3^+$ T cells were isolated from spleen by negative selection using the mouse T cell enrichment kit from R & D Systems. The cells were resuspended at a concentration of 10^7 cells per ml in HBSS, and chemotaxis in response to various ligands was determined by loading individual chambers of a 48-well chemotaxis apparatus (Nuclepore) with 50 μl of cells. The chamber was incubated at 37°C for 1 hr. Migrating cells adhering to the bottom of the membrane separating the chambers were stained with Diff-Quick (Fisher) and counted under 400× magnification. The cells in five random fields were counted, noting the differential percentages of mononuclear cells, neutrophils, and eosinophils.

RESULTS

The PLC β2 gene was disrupted as shown in Fig. 1*A*. The X and Y boxes represent conserved amino acid sequence motifs found in all members of the PLC family, and they comprise the catalytic domain responsible for PLC activity (22). The exon of the PLC β2 gene, which encodes part of the X box, was disrupted by the insertion of a neomycin-resistance gene-expression unit. Homologous recombinants that maintained the null mutation in the PLC β2 gene were detected by PCR with the primers shown in Fig. 1*A*, and the genomic structure

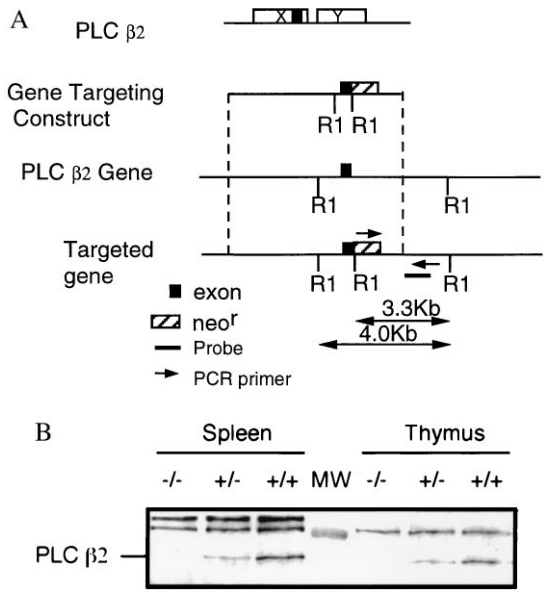

FIG. 1. Generation of PLC β2-null mice. (*A*) Gene-targeting construct and the homologous recombination process. R1, *Eco*RI restriction site. (*B*) Western analysis of mouse tissues with a PLC-β2-specific antibody. Spleen and thymus homogenates from the wild type (+/+), heterozygous (+/−), and homozygous (−/−) mice were separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and detected with a PLC β4-specific antibody (Santa Cruz Biotechnology). MW is a molecular weight standard of 200,000.

was confirmed by Southern analysis (data not shown). No PLC β2 protein was detected with a PLC β2-specific antiserum in homogenates from homozygous PLC β2-deficient mice (Fig. 1*B*).

The homozygous deficient mice show no apparent phenotypes at the systemic and cellular levels; PLC β2-deficient mice are similar to wild-type littermates in body weight, survival rate, appearance, and behavior. Flow cytometric analyses did not reveal any significant changes in populations of $CD3^+$, $CD4^+$, $CD8^+$, IgM^+, $CD45^+$, Gr-1, $MAC\text{-}1^+$, and $Mac\text{-}3^+$ cells derived from spleen, thymus, and bone marrow. Differential leukocyte counts of cells with morphologies corresponding to mononuclear cells, eosinophils, and neutrophils in bone marrow, peripheral blood, and peritoneal cavity lavage were similar in PLC β2-deficient and wild-type mice. Therefore, PLC β2 does not appear to be required for production of leukocytes.

To understand the involvement of PLC β2 in chemoattractant-induced responses, we investigated how the deficiency affects inositol phosphate accumulation, superoxide production, elevation of intracellular Ca^{2+}, and chemotaxis in response to chemoattractants. The fMLP-induced accumulation of inositol phosphates in neutrophils isolated from the bone marrow of PLC β2-null and wild-type mice was determined. As shown in Table 1, fMLP induced significant increases in the levels of inositol phosphates in neutrophils from wild-type mice, and the response was sensitive to PTx. However, fMLP-induced accumulation of inositol phosphates was significantly reduced in neutrophils isolated from the PLC β2-null mice, although the levels of inositol phosphates remained at 20% of +/+ levels. Given the absence of PLC β2 protein (Fig. 1*B*), the residual activity is likely the result of other PLC β isoforms and/or PLC γ isoforms that are regulated by fMLP. In any event, it is clear that PLC β2 is the major isoform that mediates the PTx-sensitive accumulation of inositol phosphates induced by fMLP.

fMLP-mediated Ca^{2+} release, superoxide production, and up-regulation of MAC-1 expression in neutrophils were also investigated. In neutrophils lacking PLC β2, there was only about a 30% increase in fMLP-induced intracellular Ca^{2+} compared with neutrophils derived from wild-type mice (Table 1). Similar results were also observed with IL-8- and MIP-1α-induced activation (data not shown). We also found that fMLP (Table 1), IL-8, and MIP-1α (data not shown)-induced superoxide production and up-regulation of cell surface expression of MAC-1 were significantly attenuated in neutrophils lacking PLC β2 (Table 1). In contrast, lipopolysaccharide-induced superoxide production was not affected in neutrophils lacking PLC β2 (data not shown). Thus, the mechanism for the induced response is intact, but coupling to the chemoattractant receptors is lost in these cells. We conclude that PLC β2 plays a central role in specific chemoattractant-induced superoxide production and up-regulation of MAC-1 as well as in chemoattractant-induced Ca^{2+} release and inositol trisphosphate formation in neutrophils.

One of the major biological responses mediated by chemoattractants is chemotaxis of leukocytes along a gradient of attractant (23). fMLP- and IL-8-induced chemotaxis activities were assessed using leukocytes isolated from bone marrow of PLC β2-null and wild-type mice in Boyden chamber assays. Both ligands attracted only neutrophils as judged on the basis of the morphology of the migrating cells bound to the membrane after differential staining. It is clear that IL-8- and fMLP-induced chemotaxis of PLC β2-deficient neutrophils was not impaired (Fig. 2 *A* and *B*). On the contrary, neutrophils derived from the PLC β2-null mice were consistently found to be slightly more sensitive to lower concentrations of fMLP and IL-8 in the chemotaxis assays (Fig. 2 *A* and *B*). MIP-1α-induced chemotaxis of bone marrow leukocytes was also investigated. MIP-1α attracted both mouse polymorphonu-

Table 1. fMLP-elicited responses in murine neutrophils

Mice	Inositol phosphates, dpm*		Ca^{2+}, nM†		Superoxide, pmol/min	MAC-1, %‡
	− PTx	+ PTx	− PTx	+ PTx		
PLC β2 +/+	1,247 ± 94	137 ± 50	101 ± 15	ND	13.8 ± 2.1	100
PLC β2 −/−	239 ± 89	120 ± 75	29 ± 4	ND	1.66 ± 0.5	25.2 ± 10.2

Data are shown as mean ± SEM. At least three independent experiments were performed. ND, not detected.
*The basal level of inositol phosphates is 898 ± 110 dpm.
†The resting Ca^{2+} concentration is 84.2 ± 8.9 nM.
‡Data are expressed as a percentage of the +/+ levels.

clear and mononuclear cells. Once again, cells lacking PLC β2 were more effectively recruited by MIP-1α-induced chemotaxis than those containing PLC β2 (Fig. 2*C*). In particular, MIP-1α-induced chemotaxis of mouse eosinophils was greatly enhanced in the absence of PLC β2 (Fig. 2*C*). The mononuclear cells attracted by MIP-1α included both monocytes and lymphocytes. To investigate the chemotaxis of lymphocytes, we isolated $CD3^+$ T cells from spleen. We tested both MIP-1α and RANTES as chemoattractants that are known for their ability to induce activity in T lymphocytes (13–15). Both ligands induced stronger chemotaxis activity in cells lacking PLC β2 (Fig. 2 *D* and *E*). The study was also extended to eotaxin-induced chemotaxis of eosinophils. Eotaxin, like the other chemoattractants, elicited stronger chemotaxis activity from eosinophils that lacked PLC β2 than from wild-type cells (Fig. 2*F*). In summary, deficiency in the PLC β2-linked pathway does not decrease the chemotaxis response as measured by the Boyden chamber assay; instead the loss of the PLC β2 pathway augments chemotaxis. The observations of enhanced chemotaxis were confirmed using an *in vivo* model of leukocyte recruitment. Mice were sensitized with two subcutaneous injections of methylated bovine serum albumin (MBSA) or saline solution over a 2-week period, followed 1 week later by an intrathoracic injection of MBSA. The day following this challenge the thoracic cavity was flushed with sterile saline containing heparin. The total number of nucleated cells and the fraction of eosinophils in the thoracic cavity of the MBSA-immunized mutant and wild-type mice were compared. Approximately 4 times as many eosinophils ($5.4 \pm 1.2 \times 10^5$) were recruited in the PLC β2-null mice ($n = 7$) as in the wild-type animals ($1.5 \pm 0.4 \times 10^5$, $n = 5$). These data are consistent with the *in vitro* results and demonstrate that eosinophils derived from PLC β2-null mice have enhanced chemotactic responses.

The potential systemic effects of the PLC β2 deficiency were assessed by using two models of infection. Bacterial infection was achieved by injecting mice with ampicillin-resistant bacteria. The resulting peritonitis was assessed the following day as a function of the number of surviving bacteria recovered from a peritoneal cavity lavage. The number of bacteria that

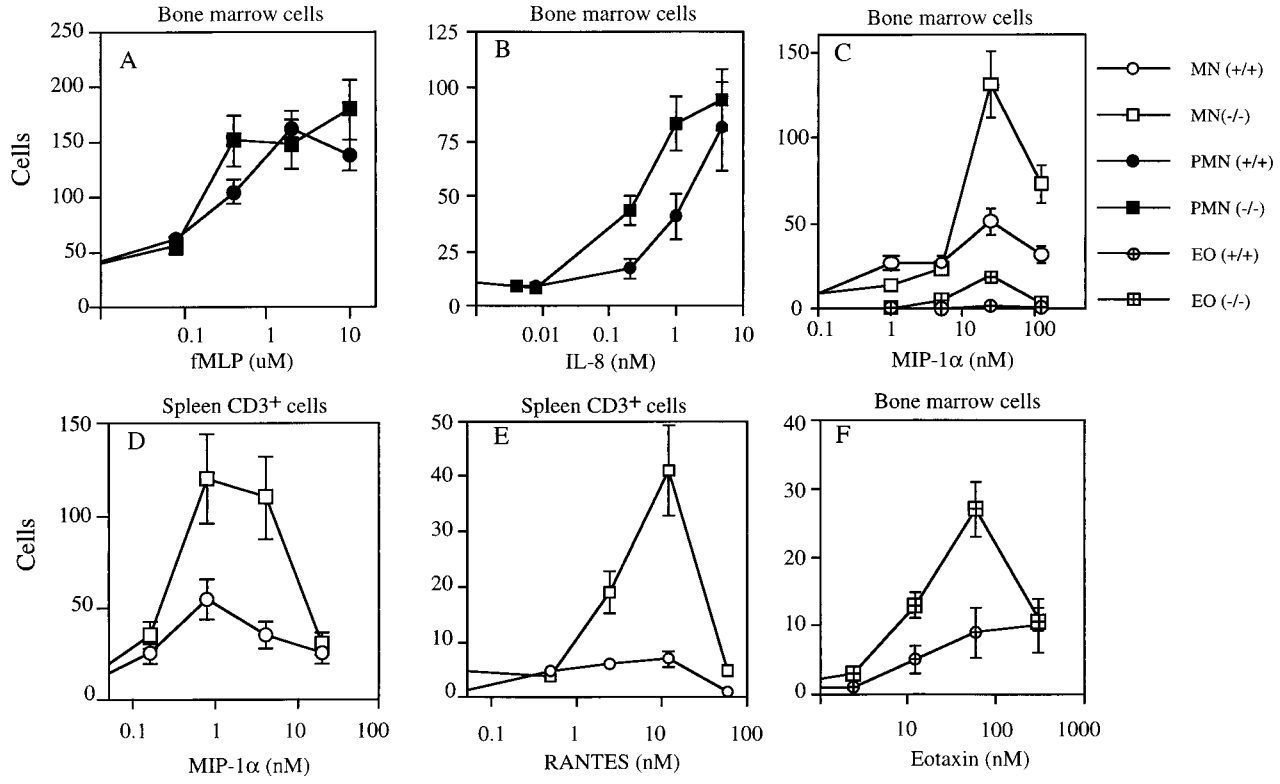

FIG. 2. Chemoattractant-elicited chemotaxis. Bone marrow cells (*A*, *B*, *C*, and *F*) and $CD3^+$ T cells (*D* and *E*) were loaded into individual chambers of a 48-well chemotaxis apparatus. The chamber was incubated at 37°C for 1 hr. Migrating cells adhering to the bottom sides of the membrane separating the chambers were stained with Diff-Quick (Fisher) and counted. The cells in five random fields were counted, noting the differential percentages of mononuclear cells (MN; open symbols), neutrophils (PMN; filled symbols), and eosinophils (EO; crossed symbols). Cells isolated from PLC β2-null mice and from the wild-type mice are represented by squares and circles, respectively. The data are presented as mean ± SEM.

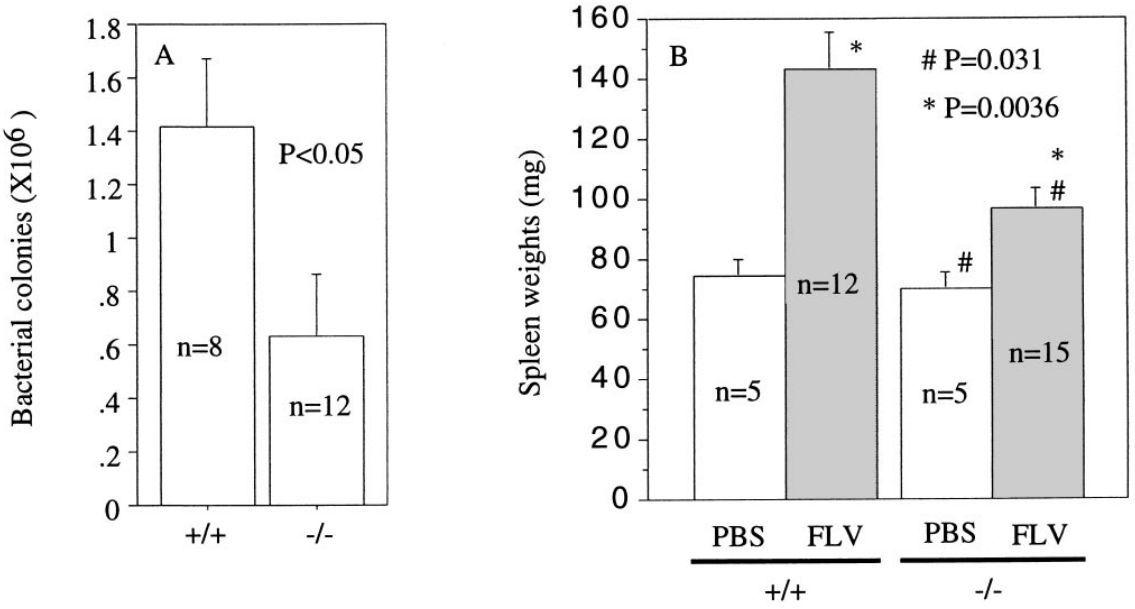

FIG. 3. Host responses to infections. (*A*) Peritonitis. DH5α *Escherichia coli* bacteria (1×10^8) containing an ampicillin-resistance plasmid were injected into the peritoneum. The following day exudate cells were recovered by lavage of the peritoneal cavity with 5 ml of PBS. White blood cells were collected by centrifugation and treated with 0.1% Triton X-100 to release ingested bacteria. Recovered bacteria were diluted and plated on an ampicillin-containing plate, and the number of colonies was counted. (*B*) Viral infection. Friend leukemia viruses (FLV; 1×10^3 virons per mouse) or PBS was injected intraperitoneally. Spleen size was assessed by weight after 14 days. The data were presented as mean ± SEM. The *P* value was calculated from comparison of the two genotypes by a nonparametric analysis of variance for unrelated samples (Mann–Whitney test).

survived in PLC β2-null mice was less than in the wild-type mice (Fig. 3*A*). This result suggests that the PLC β2 deficiency does not compromise the host's ability to deal with the bacterial challenge. The mice were also challenged with Friend leukemia virus (FLV), which induces splenomegaly (24). FLV was injected peritoneally, and 2 weeks later the spleens were weighed. The weights of wild-type spleens infected with FLV were double those of noninfected, but FLV induced only slight increases in spleen weight in the PLC β2-null mice (Fig. 3*B*). There is a clear statistical difference between the weight of the PLC β2-null spleens and those of wild type after treatment with FLV. This difference together with the difference in the bacterial survival rate suggests that disruption of the PLC β2-linked pathway does not impair, and may even enhance, the *in vivo* response to bacterial and viral exposure.

DISCUSSION

Our data delineate two apparently different functions for PLC β2 in responses mediated by chemoattractant receptors. PLC β2 deficiency results in a significant reduction of chemoattractant-induced inositol phosphate accumulation, intracellular Ca^{2+} levels, superoxide production, and cell surface MAC-1 expression. However, leukocytes lacking PLC β2 showed enhanced chemoattractant-induced chemotaxis. These results suggest that specific immediate molecular responses to chemoattractants are mediated through PLC β2 signaling, but more complex responses such as chemotaxis are regulated by inputs from a number of pathways. Our data suggest that PLC β2 may be part of a negative pathway that attenuates chemotaxis. Previous reports (25–29) have implicated protein kinase C (PKC) in desensitization of G protein-mediated signal transduction, including responses to fMLP, IL-8, and platelet-activating factor. PLC β2 may be a required component of this desensitization pathway. In addition, the role of PLC β2 is cell-specific, since PLC β2-null leukocyte populations differentially responded to chemoattractants (Fig. 2). Our results also suggest that PLC β2-mediated superoxide production and up-regulation of MAC-1 in neutrophils may not be essential for the host's response to bacterial challenges. We do not know if the enhanced chemotaxis observed in the leukocytes is the cause of the apparent increase in resistance to viral challenge. Nevertheless, it is clear that PLC β2 plays a role in leukocyte functions at the cellular and at the whole animal level. The PLC β2 mice provide a means to analyze the contributions of various signal transduction pathways to complex cellular and tissue functions *in vivo*.

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